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Tumor suppressor function of WT1 in acute promyelocytic leukemia

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analysis; LDW performed data analysis; SMR performed data analysis; CAM performed data analysis; CDSK performed experiments; HRL performed experiments; TTE performed experiments; VB performed experiments; MG performed experiments; AJM performed experiments; TJL supervised the study and wrote the manuscript
Originally identified as a cancer susceptibility gene, Wilms’ Tumor 1 gene \( (WT1) \) is overexpressed or mutated in a wide variety of malignancies, including Acute Myeloid Leukemia (AML). \( WT1 \) is a zinc-finger transcription factor comprised of C-terminal zinc-finger DNA binding domains and an N-terminal transactivation domain thought to regulate interactions with partner proteins. Germline \( WT1 \) mutations consist primarily of nonsense mutations that truncate the C-terminal domains, or missense mutations that disrupt DNA binding, and these mutations result in both developmental abnormalities and predisposition to Wilms’ tumor.\(^{(1)}\)

In normal human CD34+ hematopoietic stem/progenitor cells (HSPCs), wild type \( WT1 \) is expressed at a low level, but it is highly expressed in nearly all cases of AML. Among all AML subtypes, \( WT1 \) expression is generally highest in Acute Promyelocytic Leukemia (APL/M3 AML), the AML subtype initiated by the \( PML-RARA \) fusion gene (Supplementary Figure 1A, 1D).\(^{(2, 3)}\) In addition, we and others \(^{(4)}\) have identified recurrent \( WT1 \) mutations in APL cases (11/42 [26%] in this study) (Supplementary Figure 1C). In 7/11 of these cases, \( WT1 \) mutations occurred at a significantly lower variant allele frequency (VAF) than \( PML-RARA \), suggesting they are cooperating events in subclones (data not shown). \( WT1 \) mutations have been associated with worse prognosis in non-M3 AML, although no such association has been shown in APL. The spectrum of \( WT1 \) mutations is similar in APL vs. other AML cases (Supplementary Figure 1B), suggesting that \( WT1 \) mutations may have similar biologic activities across all AML subtypes.

These observations frame a well-known--but unsolved--paradox that we attempt to address here: does a high level of wild type \( WT1 \) expression contribute to the initiation or progression of AML/APL, or conversely, does it reflect a tumor suppressor activity, since inactivating mutations appear to contribute to disease progression?

To explore these questions, we first tested the ability of \( Wt1 \) mutations to cooperate with \( PML-RARA \) in a well-characterized murine APL model. \( Ctg-PML-RARA \) mice express the PML-
RARA fusion cDNA in immature hematopoietic progenitor cells, and succumb to an APL-like disease with a latency of about 1 year in C57Bl/6J mice.(5) To test whether Wt1 mutations can cooperate with PML-RARA in this model, we used CRISPR/Cas9 to generate indels in Wt1 exon 8 or, as a control, the Rosa26 locus. Since murine Wt1 is highly homologous to the human protein, these mutations should mimic those commonly found in APL patients. Despite efficient mutation generation in Ctsg-PML-RARA bone marrow cells (Supplementary Figure 2A), there was no survival difference between mice transplanted with Ctsg-PML-RARA cells with Wt1 mutations vs. Rosa26 mutations (Supplementary Figure 2C). We therefore evaluated APL tumors arising in these mice, and observed that tumors could arise either from wild type or mutant Wt1/Rosa26 progenitors (Supplementary Figure 2B). Surprisingly, we did not detect Wt1 protein in these tumors by western blotting (data not shown). Similarly, in a panel of 16 previously banked murine APL tumors from Ctsg-PML-RARA mice, RNA sequencing revealed virtually undetectable levels of Wt1 mRNA (Supplementary Figure 2D), in contrast to the high expression seen in human APL samples. Together, these data suggest that important interspecies differences in Wt1 regulation and function are important for the lack of a phenotype in this mouse model.

We next evaluated WT1 expression in human CD34+ cells by transducing umbilical cord blood-derived CD34+ cells with retroviruses encoding PML-RARA, RUNX1-RUNX1T1, or MYC; RUNX1-RUNX1T1 and MYC have previously been shown to confer self-renewal and expansion of human HSPCs in vitro and in xenograft models, and therefore act as positive controls.(6, 7) Consistent with previously reported results, we found that human HSPCs transduced with RUNX1-RUNX1T1 and MYC expand robustly over two weeks in culture, while HSPCs transduced with PML-RARA expand more slowly (data not shown). To test whether WT1 expression is affected by transduction with these retroviral constructs, GFP+ cells were sorted 7 days after transduction, RNA was isolated, and WT1 expression was measured by quantitative
RT-PCR. Figure 1A shows upregulation of WT1 mRNA in sorted human HSPCs transduced with PML-RARA, RUNX1-RUNX1T1, and MYC (6 to 18-fold increase, p<0.05 for PML-RARA and MYC). WT1 protein abundance also increased dramatically in the same cells during this timeframe (Figure 1B). To identify other genes dysregulated by PML-RARA transduction, we transduced both mouse and human HSPCs with GFP-tagged retroviruses encoding PML-RARA or an empty vector, as has been previously reported (N=2 and N=3 separate experiments for human and mouse cells respectively).(8, 9) Seven days after transduction, GFP+ cells were sorted and bulk RNA sequencing was performed to identify differentially expressed genes (DEGs, 5,347 identified for mouse samples, and 1,885 identified for human samples, Figure 1C). There was significant overlap between orthologous mouse and human DEGs after transduction with PML-RARA (Figure 1D, p=9.6 x 10^{-118} based on the Hypergeometric Test); further, of 867 overlapping orthologues, 82% were coordinately regulated. However, while WT1 was ~13-fold upregulated in human cells transduced with PML-RARA, Wt1 expression was extremely low and did not increase in murine cells transduced with PML-RARA (Figure 1E), validating the interspecies difference in WT1/Wt1 regulation noted above.

GFP+ PML-RARA-expressing human cord blood cells expanded modestly during the first weeks of culture, but increased dramatically 3-4 weeks after transduction (Supplementary Figure 3A). After 6 weeks in culture, they resembled primary APL cells morphologically and immunophenotypically (Supplementary Figure 3 B-C). In addition, they were sensitive to treatment with all-trans retinoic acid (ATRA), a hallmark of APL cells (Supplementary Figure 3D). Transduced cells were not immortalized, as they stopped proliferating around 8-9 weeks after initiation, and they fail to engraft immunodeficient mice (data not shown). Given its reported role as a tumor suppressor in other cancer types, we hypothesized that this upregulation may reflect an attempt of WT1 to suppress the proliferative response induced by PML-RARA, similar to the increased TP53 activity observed in cells responding to genotoxic
However, as noted above, it is also possible that high WT1 expression actively promotes the growth or survival of APL cells. To distinguish between these possibilities, we performed WT1 overexpression vs. loss-of-function experiments in PML-RARA-transduced cord blood cells.

First, we used lentiviral vectors to overexpress the two most common isoforms of WT1 (KTS+ and KTS-) in human CD34 cells in culture. In the absence of other cooperating oncogenes, WT1 overexpression led to the rapid disappearance of transduced cells (Figure 2A), consistent with previous reports that WT1 overexpression causes differentiation and death of CD34+ cells.(11) Next, we asked whether inactivating mutations in WT1 could enhance expansion of CD34+ cells expressing PML-RARA. We transduced CD34+ cells with PML-RARA or empty vector, and 2 days after transduction used CRISPR/Cas9 to generate inactivating indels in WT1, or as a control, AAVS1. After 4-8 weeks in culture, GFP+ cells were sorted and the frequency of WT1 or AAVS1 indels in GFP+ cells at the end of the culture period was compared to the frequency at the beginning. WT1 mutations were selected for over time, and were significantly increased in PML-RARA-transduced cells 4-8 weeks after the WT1 mutations were introduced (Figure 2B, bottom panels). In contrast, cells containing mutations in AAVS1 did not increase in frequency (Figure 2B, top panels). Overall cell numbers significantly increased in cells bearing both PML-RARA and WT1 mutations, compared to cells with PML-RARA and AAVS1 mutations, or cells transduced with an empty vector with WT1 mutations (Figure 2C). Together, these findings suggest that WT1 inactivation enhances the growth of PML-RARA-expressing hematopoietic cells, strongly suggesting that WT1 acts as a tumor suppressor in this context.

Based on the above findings, we propose a simple model to explain these paradoxical observations: WT1 expression in HSPCs is normally activated as an adaptive and inhibitory response to oncogenic mutations that cause proliferation, a response that is intended to slow their growth. The subsequent development of inactivating WT1 mutations in some cases would
then provide a further growth advantage by removing that normal inhibitory response.

Supporting this hypothesis, we found that a) retroviral transduction of CD34+ cells with PML-RARA, RUNX1-RUNX1T1, or MYC all led to a robust induction of WT1 expression; b) forced expression of wild type WT1 by itself does not promote CD34 cell expansion; and c) inactivation of WT1 in PML-RARA-expressing CD34+ cells leads to an additional growth advantage. Although the mechanism of WT1 gene activation by oncogenes is not yet clear, high levels of WT1 expression are found in nearly all AML cases, regardless of subtype or mutational landscape. In addition, since the majority of AML/APL cases do not have WT1 mutations, a corollary of this hypothesis is that leukemias with wild type WT1 must have developed alternative means to circumvent the inhibitory pressure that WT1 induction may exert.

Finally, the downstream mechanisms by which WT1 mutations lead to a growth advantage in AML cells are currently unclear, and may depend on the context of the cooperating mutations. In addition to its well-described function as a locus-specific transcription factor(12), recent studies have suggested that WT1 mutations may cause epigenetic changes via effects on DNA methylation and interactions with TET family methylcytosine deoxygenases.(13-16) A better understanding of how WT1 mutations activate these pathways in AML cells will be required to fully exploit their potential therapeutic value.
References:


**Figure Legends:**

**Figure 1.** *WT1* expression is induced in human CD34+ cells transduced with *RUNX1-RUNX1T1, PML-RARA, or MYC.* Umbilical cord blood-derived CD34+ cells were cultured in cytokines after transduction with GFP-tagged retroviruses expressing *RUNX1-RUNX1T1, MYC, PML-RARA,* or an empty vector control. (A) *WT1* expression is induced in CD34+ cells transduced with *RUNX1-RUNX1T1, MYC,* or *PML-RARA* compared to controls transduced with empty vector (GFP, green) or untransduced cells (CD34, pink). CD34+ cells transduced with each vector (N=2-6 separate experiments) were cultured for 7 days, RNA was isolated from flow-sorted GFP+ cells, and *WT1* mRNA was quantified by real time RT-PCR. P values were calculated using Student’s t test. (B) Western blot showing expression of WT1 in CD34+ cord blood cells 7 days after transduction with *RUNX1-RUNX1T1* (AE), *PML-RARA* (PR), *MYC,* empty vector (GFP), or untransduced (CTRL). Lysates were made from sorted GFP+ cells except CTRL, which was made from equivalent cell numbers of untransduced cells cultured in parallel. Blot represents one of three representative experiments. (C) Heatmaps showing differentially expressed genes (DEGs) in human or mouse cells transduced with a *PML-RARA*-expressing MSCV vector. Human CD34+ cells or mouse lineage-depleted bone marrow cells were transduced with IRES-GFP-tagged retroviruses containing a *PML-RARA* cDNA, or no insert (Empty Vector). After 7 days in culture, GFP+ cells were flow sorted and RNA was isolated for RNA-seq. DEGs were identified using a false discovery rate (FDR) cutoff of <0.05 after filtering out genes with low expression across all samples (see Supplementary Methods). Heatmaps show DEGs in *PML-RARA* versus empty vector-transduced human (N=2 separate experiments) and mouse (N=3 separate experiments) progenitor cells. (D) Venn diagram showing overlap in orthologous mouse and human DEGs from (C). Of 4915 mouse DEG having human orthologues, 867 are DEGs in the analysis of human genes (p=9.6 x 10^{-118} using the Hypergeometric Test). (E) *WT1* expression is increased by *PML-RARA* transduction in human CD34+ cells (left panel), but not in mouse bone marrow-derived cells (right panel).
WT1/Wt1 expression values from the RNA-seq experiment described above are shown (C). DEGs were identified using a false discovery rate (FDR) cutoff of <0.05 after filtering out genes with low expression across all samples. P values were calculated using Student’s t test. CPM, counts per million.

Figure 2. Inactivating mutations in WT1 provide a growth advantage for PML-RARA-transduced CD34+ cells. (A) Umbilical cord blood-derived CD34+ cells were transduced with GFP-tagged lentiviruses encoding the two most common WT1 isoforms (KTS+ and KTS-), or an empty vector. Cells were maintained in culture with cytokines, and GFP+ cells were quantified at different time points. Shown are percent GFP+ cells over time in WT1 (right) or empty vector (left) transduced cultures normalized for transduction efficiency at beginning of the culture period (N=4 individual experiments). Black dotted lines show line of best fit calculated by linear regression. Transduction with WT1 isoforms (KTS+ and KTS-) leads to loss of GFP+ cells (slope b=-1.18 per day, p<0.001), while empty vector-transduced cells have GFP+ cells throughout the culture period (slope b=-0.54, p=0.32). P values were calculated using a linear regression model, and represent the probability that the slope of the best fit line equals zero. (B) Human CD34+ cord blood cells were transduced with PML-RARA-expressing retrovirus or empty vector, and 48 hours later CRISPR/Cas9 was used to generate mutations in WT1 (exon 1 or exon 8) or AAVS1 (a negative control locus). GFP+ cells were sorted at different time points from cultures that had been transduced with a vector containing PML-RARA (right panels) or no insert (empty vector, left panels). DNA was isolated and PCR products containing the gRNA target sites were digitally sequenced to determine the precise variant allele frequencies of mutations in WT1 (bottom panels) or AAVS1 (top panels). Shown are change in variant allele frequency (VAF) of AAVS1 mutations or WT1 mutations over time (N=3-6 separate experiments). Mutations in WT1 exon1 are shown in green, or WT1 exon 8 in red. Black dotted lines show line of best fit calculated by linear regression. Cells containing mutations in WT1...
show a trend toward expansion in empty vector-transduced CD34+ cells (slope b=0.003 increase per day, p=0.20), and a statistically significant expansion in PML-RARA-transduced cells (slope b=0.006 increase per day, p=0.007). In contrast, cells with mutations in AAVS1 do not expand over time. P values were calculated using a linear regression model, and represent the probability that the slope of the best fit line equals zero. (C) Increase in overall cell numbers in cultures transduced with GFP (left) or PML-RARA (right). P values were calculated using Student’s t-test.
Figure 1.

A. WT1 qRT-PCR

B. Western Blot

C. Human DEG (N=1885) and Mouse DEG (N=5347)

D. Venn Diagram

E. Comparison of TPM values for WT1 expression in GFP and PR viruses in human and mouse models.
Figure 2.

A. Empty Vector

WT1 overexpression

b = -0.54
p = 0.32

b = -1.18
p < 0.001

Day

Relative GFP proportion

Day

Relative GFP proportion

b = -0.00045
p = 0.85

b = -0.003
p = 0.24

Day

VAF

Day

VAF

empty vector + Control AAVS1

empty vector + WT1

PR + Control AAVS1

PR + WT1 mutations

b = 0.003
p = 0.20

b = 0.006
p = 0.007

Day

VAF

Day

VAF

b = 0.003
p = 0.24

b = 0.00045
p = 0.85

Day

VAF

Day

VAF

C.

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Supplementary Figures and Legends

Supplementary Figure 1.
Supplementary Figure 1. *WT1* is both highly expressed and frequently mutated in APL.

(A) RNA expression data from the AML TCGA dataset showing expression of *WT1* across all FAB subtypes. *WT1* expression is significantly higher in M3 AML compared to all other subtypes with p-values ranging from 0.04 (vs. M7) to $2.43 \times 10^{-13}$ (vs. M5) using Tukey’s Range Test. TPM, Transcripts Per Kilobase Million. (B) Schematics showing *WT1* mutations in AML samples from the TCGA dataset (N=200 cases, top) and in the APL cases from this study (N=42 cases, bottom). Green circles represent nonsense or frameshift mutations, red circles represent missense mutations, numbers indicate exon number, ZF1-ZF4 are zinc finger domains 1-4. Hotspot mutations (e.g. R394Q/W) are labelled. (C) Plot showing gene mutations co-occurring with the *PML-RARA* translocation in 42 cases of APL. Yellow boxes indicate mutations that co-occur within a biologic category and black inset boxes indicate mutations that occur in more than one allele. Blue boxes indicate cases where no cooperating mutations were identified with a capture panel containing the recurrently mutated genes in the TCGA AML study (2). (D) RNA expression data from the AML TCGA dataset showing expression of *WT1* in WT1 mutant (first and third bars) compared to WT1 wild type cases (second and fourth bars). Cases are stratified as APL (two left bars) or non-M3 (right two bars). WT1 expression is significantly lower in the non-M3, WT1 wild-type cases as a group (p<0.01 for all comparisons using Tukey’s Range Test.) TPM, Transcripts Per Kilobase Million.
Supplementary Figure 2
Supplementary Figure 2. *Wt1* loss-of-function mutations do not cooperate with *PML-RARA* in a mouse APL model. (A) Amplicon sequencing of the gRNA target regions from *Ctsg-PML-RARA* lineage depleted bone marrow cells after CRISPR editing, prior to transplantation into lethally irradiated recipients. Shown is the mutational spectrum of hematopoietic cells edited at *Rosa26* (left) or *Wt1* (right) loci. Bolded missense variants are presumed to be PCR artifacts generated during amplification. (B) Mice that died during the tumor watch succumbed to clonal APL arising from either a mutated or an unmutated hematopoietic progenitor cell. DNA was isolated from unfractionated spleen cells from mice with viable tissue at the time of death (N=8), PCR was performed using primers flanking the *Rosa26* or *Wt1* CRISPR gRNA site, and mutant reads were quantified by digital sequencing. Shown is the proportion of wild type to mutant *Rosa26* (left) or *Wt1* (right) reads in each sequenced sample. These APLs all contained cells without *Wt1* mutations, suggesting that these cells were not selected for in this model system. (C) Survival of mice (N=15-18 recipients in each group) transplanted with BM from *Ctsg-PML-RARA*+/− knock-in mice genetically altered using CRISPR/Cas9 to create indels in *Wt1* (blue) or control mutations in *Rosa26* (red). The log rank test for difference in survival gives a p-value of p = 0.47. (D) RNA expression of selected transcription factors in 16 previously banked mouse APL tumors. Red box highlights *Wt1* expression.
Supplementary Figure 3
Supplementary Figure 3. Human CD34+ cells transduced with *PML-RARA* have a growth advantage in long term culture, and phenotypically resemble APL cells. Umbilical cord blood-derived CD34+ cells were transduced with a GFP-tagged retrovirus expressing *PML-RARA* and cultured in cytokines for up to 8 weeks. (A) Expansion of *PML-RARA* (right) or empty vector-transduced (left) human CD34+ cells *in vitro*. Shown is percent of GFP+ cells in culture versus time. Black dotted lines show line of best fit calculated by linear regression. Transduction with *PML-RARA* leads to expansion of GFP+ cells (slope b=1.3% per day, p<0.001), while empty vector-transduced cells gradually decrease in culture (slope b=-0.05, p<0.001). P values were calculated using a linear regression model, and represent the probability that the slope of the best fit line equals zero. (B) Representative flow cytometry plots showing GFP positive and negative populations in a culture of *PML-RARA*-transduced CD34+ cells maintained in culture for 5 weeks (top plot). Other plots show KIT vs GFP, CD13 vs CD11b, CD33 vs CD14, CD56 vs HLA-DR, and CD18 vs empty channel gated on the GFP positive (right) or GFP negative (left) populations. (C) Representative photomicrographs of CD34+ cells expressing *PML-RARA* (bottom) or empty vector (top) after 6-8 weeks of culture.
Empty vector-transduced cells display a full range of maturing myeloid cells (top), while cultures with \textit{PML-RARA}-transduced cells (bottom) contain predominantly promyelocytic cells with primary granules overlying the nucleus, consistent with a myeloid maturation arrest. (D) \textit{PML-RARA}-transduced CD34+ cells are sensitive to all-trans retinoic acid (ATRA) \textit{in vitro}. \textit{PML-RARA}-transduced CD34+ cells were expanded 6 weeks in culture and then treated \textit{in vitro} for up to three days with ATRA or vehicle. Shown is total cell number on each day of treatment. (E) RNA sequencing showing relative level of WT1 expression 7 days after lentiviral transduction of CD34+ cells with KTS+ isoform of WT1 (“WT1”, green bar) compared to cells transduced with PML-RARA or empty vector (“PR” and “GFP”, red and blue bars). N=2 experiments each construct. p<0.05 in both comparisons comparisons by Student T test. TPM, transcripts per kilobase million.